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Electrostatic-mediated enhancement of protein antigen immunogenicity using charged TLR2-targeting lipopeptides

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Abstract

The low immunogenicities exhibited by most soluble proteins are in general due to the absence of any molecular signatures that are recognized by the immune system as dangerous. We show here that *electrostatic binding* of synthetic branched cationic or anionic lipopeptides that contain the TLR2 agonist Pam_2Cys can markedly enhance protein immunogenicity. High protein-specific antibody titres in animals were achieved by vaccination with formulations containing lipopeptide and protein of opposite charge. This response was not totally dependent on electrostatic binding because vaccination with similarly charged constituents also resulted in the induction of strong, albeit lower, antibody titres. The induction of $CD8^+$ T cell-mediated responses, however, was achieved only by vaccination with formulations containing lipopeptide and protein of opposite but not similar charge. These responses also correlated with the ability of the electrostatically associated lipopeptide to facilitate dendritic cell uptake of protein antigen and trafficking into the draining lymph node. Vaccination subsequently resulted in faster viral clearance upon pulmonary infectious challenge with a chimeric influenza virus. The improvement in protein antigen immunogenicity obtained by mixing with lipopeptide led to a *99% reduction* in lung viral titres and correlates with the presence of substantial numbers of antigen-specific $CD8^+$ T cells in lung bronchoalveolar washes.

Keywords: TLR2; antibody; CD8+ T cell responses; influenza virus; protein immunogenicity; lipopeptide

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1. Introduction

Generally speaking, soluble protein antigens when administered in the absence of adjuvants or through the use of sophisticated delivery modalities are poorly immunogenic as measured by their inability to elicit antibody and especially $CD8^+$ T cells. This shortcoming requires their co-administration with an adjuvant in order to elicit an immune response. Of the many experimental adjuvants currently available, only squalene oil water emulsions and aluminum-based salt adjuvants have so far been approved for human use [1, 2]. The utilization of these adjuvants is in most cases, however, limited by the fact that although they may be effective at eliciting humoral responses, they are often ineffective at stimulating cell-, particularly $CD8^+$ T cell, mediated immunity.

Antigenic receptors that are expressed by dendritic cells (DCs), such as the Toll-like receptor (TLR) family, have evolved to recognize a diverse range of microbial components. The engagement of TLR by their appropriate ligands or agonists initiates a cascade of events that can result in the induction of antibody and cell-mediated responses and their use as "adjuvants" whether admixed- or covalently coupled to antigens, are well documented [3, 4]. With the aim of developing novel ways of adjuvanting otherwise non-immunogenic and soluble proteins, we set out in this study to investigate whether the electrostatic linkage of the TLR2 agonist dipalmitoyl-S-glyceryl-cysteine (Pam_2Cys) [5] to protein antigens as opposed to a covalent conjugation results in the enhancement of protein immunogenicity. Robust non-covalent association between protein and adjuvant would provide a manufacturing advantage compared with the chemistries involved for covalent attachment and would, furthermore, provide a simplified approach to vaccine preparation.

2. Results

1.1. Branched electrostatically charged lipopeptides

In order to facilitate firm association of Pam_2Cys with protein antigen, the lipid moiety was assembled with a branched cationic arginine $R_4(Pam_2Cys)$ or anionic glutamic acid-based structure $E_8(Pam_2Cys)$(Ref) designed to bind electrostatically to negatively charged or positively-charged antigens respectively (Figure 1A). Electrostatic association of lipopeptide and protein was demonstrated by the addition of either lipopeptide to solutions containing a constant amount of the protein antigen ovalbumin (OVA; which carries a net negative charge). Increases in the optical densities of solutions, arising from the neutralisation of charges the protein, was only demonstrated in the presence of the oppositely charged lipopeptide, $R_4(Pam_2Cys)$ and but not with $E_8(Pam_2Cys)$ lipopeptide (Figure 1B)

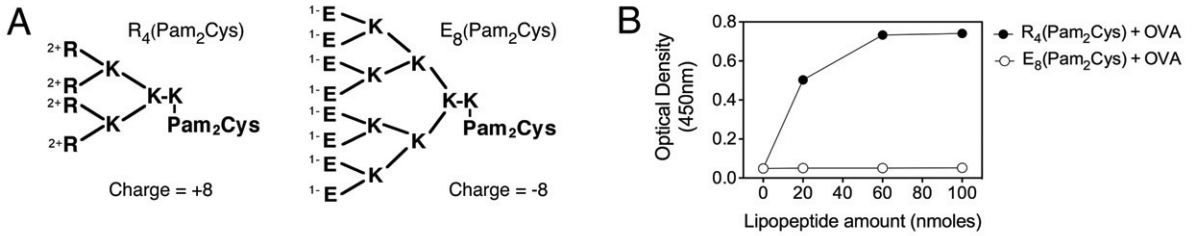

Figure 1. Schematic representation of charged lipopeptides and association with protein antigen. (A) The lipopeptide $R_4(Pam_2Cys)$ consists of a 2-tier branching lysine (K) scaffold to which are attached 4 arginine (R) residues and the lipopeptide $E_8(Pam_2Cys)$ consists of a 3-tier scaffold to which 8 glutamic acid (E) residues are attached. In each lipopeptide, the lipid moiety Pam_2Cys is attached to the ε-amino group of the C-terminal lysine. (B) Increasing amount of each lipopeptide was mixed with 1nmole of OVA in a total volume of 100µl PBS in a flat-bottom, 96-well plate. Optical densities of solutions were then measured at 450nm.

1.2. Induction of antibody & cell-mediated responses

In examining T cell responses induced by vaccination of animals with lipopeptide-protein formulations, significantly high numbers of antigen-specific cytokine producing CD8+ T cells were demonstrated in animals that received OVA formulated with $R_4(Pam_2Cys)$ but not $E_8(Pam_2Cys)$ (Figure 2A). This result was also reflected in the ability of vaccinated animals to lyse CFSE-labelled antigen-bearing target cells *in vivo* where once again $R_4(Pam_2Cys)$ but not $E_8(Pam_2Cys)$ lipopeptide was more effective (Figure 2B). The induction of antibody responses however is not totally dependent on

this association since vaccination with either lipopeptide formulated with protein could elicit similarly strong antigen-specific antibody titres (Figure 2C).

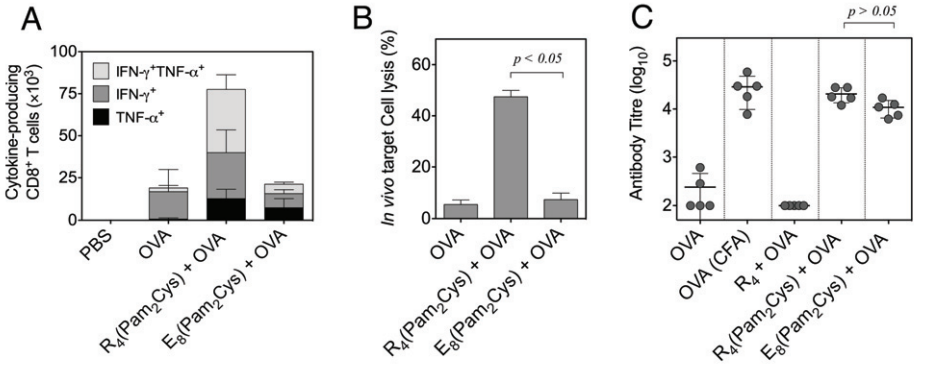

Figure 2. Induction of immune responses induced by vaccination with lipopeptides in association with protein antigen. (A) Groups of C57BL/6 mice were inoculated sub-cutaneously (s.c.) at the base of the tail with 25μg of OVA alone or preincubated with a 5-fold molar access of each lipopeptide. Spleens were removed 7 days later and IFN-γ and TNF-α secretion from OVA_{257}-specific CD8 T cells were enumerated in an intracellular cytokine-secreting assay (ICS). (B) OVA_{257}-specific in vivo lytic responses were also assessed by intra-venous injections of $CFSE^{high}$-labelled OVA_{257}-pulsed splenocytes from a naïve mouse. Spleens were removed 16 hours later and analysed for the presence of CFSE-labelled cells by flow cytometry. (C) Mice were also inoculated twice (3 weeks apart) and antibody levels in sera were determined by ELISA 2 weeks following the last dose.

1.3. Antigen uptake by dendritic cells and trafficking into draining lymph nodes

A comparison of the levels of intracellular antigen present in DCs after incubation with FITC-labelled OVA mixed with each lipopeptide showed that $R_4(Pam_2Cys)$ was considerably more effective than $E_8(Pam_2Cys)$ at improving antigen uptake (Figure 3A). This result also correlated with the ability of the lipopeptides to facilitate the trafficking of antigen-bearing dendritic cells into the draining lymph nodes of animals after vaccination (Figure 3B).

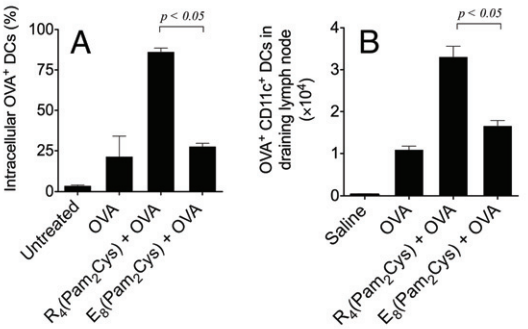

Figure 3. Uptake of lipopeptide-protein antigen and trafficking into the draining lymph. (A) Splenocyte-derived cultured DCs (2×10^5) were incubated with 5μg FITC-labelled OVA mixed with $R_4(Pam_2Cys)$ or $E_8(Pam_2Cys)$. Intracellular fluorescence was determined 24 hours later by flow cytometry after quenching extracellular fluorescence with trypan blue. (B) Groups of mice (n=3) were inoculated s.c. via the footpad with 25μg FITC-OVA mixed with either lipopeptide. Popliteal lymph nodes were obtained 24 hours later and analysed for the presence of $FITC^+$ cells.

1.4. Clearance of infection upon pulmonary challenge with chimeric influenza virus

The effectiveness of the CD8+ T cell responses elicited by vaccination using protein mixed with $R_4(Pam_2Cys)$ is best exemplified by the ability of vaccinated animals to clear pulmonary infection after challenge with a chimeric influenza virus containing the OVA_{257} immunodominant epitope in the neuraminidase stalk [6]. Only animals that were received OVA mixed with $R_4(Pam_2Cys)$ had significantly diminished viral titres in their lungs compared to those that received OVA or the lipopeptide alone (Figure 4A). Analyses of lymphocytes contained in brochoalveolar lavage washings also demonstrated that the lower lung viral titres were associated with the presence of significantly

higher numbers of cytokine-secreting antigen-specific T cells (Figure 4B).

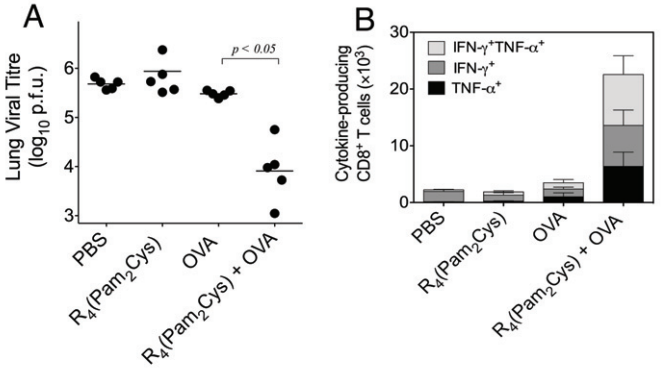

Figure 4. Induction of influenza virus clearing responses. Groups of C57BL/6 mice were inoculated twice, 14 days part, via the intranasal route with 25μg of OVA or preincubated with a 5-fold molar access of $R_4(Pam_2Cys)$. Animals were challenged with 10^4 plaque forming units of A/HK×31 influenza virus containing the OVA_{257} epitope in the neuraminidase stalk. (A) Titres of virus in lung homogenates collected 5 days after challenge were determined by plaque formation. (B) Lymphocytes present in lung bronchoalveolar lavages were also enumerated in an ICS assay for their ability to secrete cytokines in response to the presence of antigen.

2. Conclusions

From the series of experiments conducted in this study [7], we have found that the immunogenicity of an otherwise nonimmunogenic protein could be markedly enhanced if linked, not covalently, but electrostatically to a charged branched lipopeptide containing the TLR2 targeting ligand Pam_2Cys. We reason that a lipopeptide that has charged amino acids fashioned in a branched manner would provide a tentacle-like structure capable of associating electrostatically with oppositely charged portions of a protein to direct their delivery to TLR2 expressing dendritic cells. For cell-mediated responses, only inoculation with electrostatically associated lipopeptide-antigen complexes was effective at eliciting CD8+ T cells that could mediate accelerated clearance of virus from the lungs of mice challenged with influenza virus. The induction of antibody responses on the other hand was not totally dependent on this association as vaccination with similarly charged constituents also resulted in the induction of strong, albeit lower, antibody titres.

There are several advantages in using these lipopeptides to enhance protein immunogenicity in a charge dependent manner. Since most TLR ligands are non-peptidic components of infectious agents, linking them covalently to antigens may be chemically challenging whilst electrostatic association between lipopeptide and protein occurs spontaneously upon mixing. Only small amounts of lipopeptides are needed to elicit robust humoral and cell-mediated immune responses and could therefore be dose sparing and economical in use. Given the findings of this study, this approach could constitute an efficient way of inducing immune responses against soluble microbial, viral and tumorigenic protein antigens.

4. References

[1] Mesa, C., L.E. Fernandez, Challenges facing adjuvants for cancer immunotherapy. *Immunol Cell Biol*, 2004. **82**(6): 644-50.

[2] Pashine, A., N.M. Valiante, J.B. Ulmer, Targeting the innate immune response with improved vaccine adjuvants. *Nat Med*, 2005. **11**(4 Suppl): S63-8.

[3] Lahiri, A., P. Das, D. Chakravortty, Engagement of TLR signaling as adjuvant: towards smarter vaccine and beyond. *Vaccine*, 2008. **26**(52): 6777-83.

[4] van Duin, D., R. Medzhitov, A.C. Shaw, Triggering TLR signaling in vaccination. *Trends Immunol*, 2006. **27**(1): 49-55.

[5] Muhlradt, P.F., M. Kiess, H. Meyer, R. Sussmuth, G. Jung, Isolation, structure elucidation, and

synthesis of a macrophage stimulatory lipopeptide from Mycoplasma fermentans acting at picomolar concentration. *J Exp Med*, 1997. **185**(11): 1951-8.

[6] Jenkins, M.R., R. Webby, P.C. Doherty, S.J. Turner, Addition of a prominent epitope affects influenza A virus-specific CD8+ T cell immunodominance hierarchies when antigen is limiting. *J Immunol*, 2006. **177**(5): 2917-25.

[7] Chua, B.Y., D. Pejoski, S.J. Turner, W. Zeng, D.C. Jackson, Soluble proteins induce strong CD8+ T cell and antibody responses through electrostatic association with simple cationic or anionic lipopeptides that target TLR2. *Journal of immunology*, 2011. **187**(4): 1692-701.